

Article

Glutamine Synthetase1 Increases Autophagy Lysosomal Degradation of Mutant Huntingtin Aggregates in Neurons, Ameliorating Motility in a *Drosophila* Model for Huntington's Disease

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Abstract: Glutamine Synthetase1 (GS1) is a key enzyme that catalyzes the ATP-dependent synthesis of L-glutamine from L-glutamate and is also member of the Glutamate Glutamine Cycle, a complex physiological process between glia and neurons that controls glutamate homeostasis and is often found compromised in neurodegenerative diseases including Huntington's disease (HD). Here we report that the expression of GS1 in neurons ameliorates the motility defects induced by the expression of the mutant Htt, using a *Drosophila* (common fruit fly) model for HD. This phenotype is associated with the ability of GS1 to favor the autophagy that we associate with the presence of reduced Htt toxic protein aggregates in neurons expressing mutant Htt. Expression of GS1 prevents the TOR activation and phosphorylation of S6K, a mechanism that we associate with the reduced levels of essential amino acids, particularly of arginine and asparagine important for TOR activation. This study reveals a novel function for GS1 to ameliorate neuronal survival by changing amino acids' levels that induce a "starvation-like" condition responsible to induce autophagy. The identification of novel targets that inhibit TOR in neurons is of particular interest for the beneficial role that autophagy has in preserving physiological neuronal health and in the mechanisms that eliminate the formation of toxic aggregates in proteinopathies.

Keywords: Huntington's disease; glutamine synthetase1; autophagy; TOR signaling; protein aggregates; *Drosophila* model for neuronal degeneration

1. Introduction

Huntington's disease (HD) is an inherited neurodegenerative disease with a middle age clinical onset that highly depends upon the length of the CAG repeated sequence (>35) present in the first exon of the *huntingtin* gene (OMIM 143100) [1]. Mutations in the gene that expands this sequence result in a protein with a long poly-Q trait that forms toxic mHTT protein aggregates which are considered as one of the major cause for the progressive degeneration of neurons, particularly of the striatum and cortex, resulting in cognitive decline and motor defects [2–4]. Drugs as antisense oligonucleotides [5] have been recently developed to slow down the disease progression, and attention is dedicated to those that ameliorate neuronal survival by increasing autophagy to limit the formation of mHTT aggregates [6–10].

Neuronal health depends also upon maintaining glutamate at physiological levels; a process that is controlled by a sequence of biochemical reactions, called the Glutamate–Glutamine Cycle (GGC), occurring between glia and neurons that often are found altered in neuronal pathology [11]. Key components of the GGC are the enzymes Glutamine Synthetase-1 (GS1) that uses ammonia to convert glutamate into glutamine with the hydrolysis of ATP, Glutamate dehydrogenase (GDH), that converts glutamate into alpha-keto glutarate (aKG), and Glutaminase (GLS) that in neurons produces glutamate from glutamine [12]. Since the activity of GS1 was found reduced in neuronal diseases [13] and in the postmortem brains of patients with HD [14–16], we decided to investigate the contribution of GS1 to HD, using a well-established *Drosophila* model for HD that expresses the exon1 of the human *HTT* gene with 93 CAG repeats, (hereon referred to as *Htt-Q93*). This model recapitulates the cellular and molecular events described in HD, including the loss of neurons and impaired animal motility [17].

GS1 is a conserved enzyme, from bacteria to vertebrates, and in *Drosophila* there are two distinct genes, *Gs-1* and *Gs-2*, encoding for *glutamine synthetases*, both highly homologous to the human gene *GLUL* (60.5%) [18]. Here we show that the expression in neurons of *GS1* together with *Htt-Q93* significantly improves animal motility and rescues neuronal loss. At the cellular level, we found that *GS1* increases the level of autophagy, and significantly reduces the size of Htt-Q93 protein aggregates.

Autophagy is induced when amino acid levels are low, and in neurons it plays an important role for the survival and homeostasis of these post-mitotic cells, while its activation is counteracted by TOR signaling and nutrients [19]. Activation of TOR by amino acids induces the assembling of the RagA/B-C/D GTPases complex that, together with the GTPase Rheb, activates the TORC1 complex at the lysosomal membrane [20,21] to phosphorylate S6K and 4EBP target proteins [22]. Interestingly, we found that the expression of *GS1* in neurons was able to reduce the TOR signaling measured by the reduced level of S6K phosphorylation, a mechanism that was present also when *GS1* was co-expressed with Htt-Q93. Analysis of the amino acid levels in the heads of animals expressing *GS1* in neurons reveals a significant decrease of essential amino acids, including proline and arginine, known to be necessary in the mechanism of TOR activation. Finally, we show that *GS1* protein levels are reduced in human fibroblasts from HD patients, and these cells have impairment in the autophagy flux, suggesting that the role of *GS1* in the control autophagy may be conserved also in human cells.

In summary, our data suggest a novel function for *GS1* that links its activity to autophagy in neurons to the reduction of Htt-Q93 toxic aggregates in *Drosophila* HD animals. Understanding the mechanisms that link *GS1* to amino acids signaling in neurons is the initial step to comprehend a novel function for this enzyme, and of the GGC, in autophagy and neuronal survival, that is relevant not only for the control of pathological diseases such as HD, but also in the mechanisms that drive physiological neuronal health and aging.

2. Materials and Methods

2.1. Fly Husbandry and Lines

Animals were raised at low density, at 25 °C, on a standard food medium containing 9 g/L agar (ZN5 B and V, Italy), 75 g/L corn flour, 60 g/L white sugar, 30 g/L brewers' yeast (Fisher, Milano, Italy), 50 g/L fresh yeast and 50 mL/L molasses (Biosigma, Cona (Ve), Italy), along with nipagin and propionic acid (Fisher, Rodano (Mi), Italy). The following fly lines were obtained from the Bloomington *Drosophila* Stock Center: *UAS-Htt-16Q* (B33810) [23]-*GS1* is an enhancer-promoter (EP)-line with a random insertion of the *P*-element, carrying a *UAS* enhancer sequence and a basal promoter, in the *GS1* locus (B27940), *UAS-GS1RNAi* (B40836), *UAS-mCherry-Atg8a* (B37750), *elav^{c155}-Gal4* on the *X ch.* (B458) and *GMR-Gal4* (B9146). Fly lines bearing the *UAS-Htt-Q93* was a gift of from L. Marsh (University of California, Irvine), *UAS-GFP-LAMP1* and *UAS-Atg1-RNAi* where a gift from F. Parisi (Beatson Institute for Cancer Research, Glasgow, UK), *UAS-Atg5-RNAi* was a gift from T. Neufeld (University of Minnesota). EP-FOXO was a gift from Hugo Stocker (ETH-Zurich) [24].

2.2. Survival at Eclosion Analysis

The number of animals and their timing of eclosion from every cross with the *elav^{c155}-Gal4* driver were quantified, and the percentage of animals eclosed was calculated for each of the genotypes of interest with respect to the expected number of animals that did eclose.

2.3. Motility Assays

Ten larvae (3rd instar) were collected, washed with phosphate-buffered saline (PBS) and then transferred to a 14 cm Petri dish containing 1% agarose in PBS, where a visible grid was drawn to portray the arena (Supplementary Figure S4). The number of lines crossed by one larva in 1 min at room temperature was counted. Statistical analysis was performed with the Student's *t*-test; values for each genotype are represented as mean ± standard deviation (SD). Experiments were repeated at least three times. For motility assays with adult animals, 10–15 females were collected at 1 day after eclosion (DAE), transferred in a plastic vial, and their ability to climb up the empty vial after a knock-down to the bottom was analyzed, as previously described [25]. The number of flies that were able to climb half of the tube in 15 s was counted. Values were expressed as the percentage of success with respect to the total number of flies in the vial. For each genotype the test was repeated 30 times for each time point. After the test, adults were transferred in vials with food, and these vials were changed every two days. Data are represented as a curve of progressive motility impairment. The statistical analysis of variance (one-way analysis of variance (ANOVA)) was performed using PRISM GraphPad Software (version, city, CA, USA). Every experiment was repeated at least six times. The experiments were performed only with females, as in males the expression of *Htt-Q93* resulted in a low survival rate for *elav/Y; Htt-Q93/+* males that showed 50% reduction of their climbing activity at 2–3 DAE, and that were not further used (Supplementary Figure S6). The stronger effect of *Htt-Q93* in males is probably due to a dosage compensation that affects genes expressed on the *X* chromosome where the transgene *elav-Gal4* is inserted [26].

2.4. Quantification of Fluorescence (GFP) on the Adult Compound Eye

Photographs of adult eyes expressing the indicated *UAS*-transgenes in the retina, and co-expressing *UAS-GFP* using the *GMR-Gal4* promoter, were taken at 8 days after eclosion using a Leica stereomicroscope MZ10F with a fluorescent source and a camera, and by employing 5× magnification. The intensity of fluorescence in each image was quantified as the integrated density from an area containing 20 ommatidia using Adobe Photoshop-CS4, and expressed as a GFP intensity/area. At least 10 eyes (one for each animal) were used for each genotype, and these experiments were repeated twice.

2.5. Western Blot

Proteins were extracted from ten brains of larvae or from 10 adult heads collected in lysis buffer (50 mM Hepes/NaOH pH 7.4, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1.5% Triton X-100 containing phosphatases and proteases inhibitors (Roche). For the detection of the Huntingtin protein, lysates were extracted using 2% sodium dodecyl sulfate (SDS) lysis buffer (50 mM Hepes/NaOH pH 7.4, 250 mM NaCl, 1 mM EDTA, 2% SDS containing phosphatases and proteases inhibitors). Samples were sonicated three times for 10 s using a Branson Ultrasonic Sonifier 250 (Branson, Darbury, CA, USA) equipped with a microtip set at 25% power. Tissue and cell debris were removed by centrifugation at 10,000× g for 30 min at 4 °C. Proteins in the crude extract were quantified by a bicinchoninic acid (BCA) Protein assay Reagent Kit (Pierce), following the manufacturer's instructions with bovine serum albumin as the standard protein. For SDS-PAGE, samples were incubated for 8 min at 100 °C in standard reducing loading buffer; 40 µg of total protein were run on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (GE Healthcare). After blocking in 5% (*w/v*) non-fat milk in tris-buffered saline (TBS)-0.05% Tween (TBS-T), membranes were incubated overnight with primary antibodies against hHTT (1:500, Abcam ab109115), Elav (1:100, Hybridoma Bank #9F8A9), Synapsin (1:100, Hybridoma Bank #8C3), Actin5c (1:200 Hybridoma Bank #JL20), Tubulin (1:500, Chemicon, MAB3408), GABARAB/Atg8a (1:500 ABCAM ab109364), and this antibody cross-reacts with *DROSOPHILA* Atg8a [27,28], mTOR (1:500 Abcam ab45989), anti TOR-P1(1:400 gift from A. Teleman, DKFZ, Heidelberg, German), GS1 (1:800 Abcam ab64613), SQSTM1/p62 (Cell Signaling 5114), anti-*Drosophila* p70 S6K (Cell Signaling 9209), ULK1/ATG1 (1:500 Cell Signaling #D8H5), or ATG5 (1:500 Thermo Fisher #OSA00026W).

2.6. Filter Trap Assay

Ten larval brains or adult heads for each genotype were homogenized in lysis buffer (50 mM Tris- HCl pH 6.8, 2% SDS plus proteases and phosphatases inhibitors). Fifty micrograms of protein extracts were loaded on a dot-blot device and filtered through a cellulose acetate membrane (0.22 mm pore size), previously washed in 1% (*w/v*) SDS solution in PBS. After filtration, membranes were washed three times in TBS-T, saturated in 5% non-fat milk and processed for immunodetection with anti-Htt antibodies from Abcam, as described above.

2.7. Patient-Derived Cell Lines

Fibroblasts from patient and controls were derived from skin biopsies and grown in the Dulbecco's modified Eagle's medium (DMEM)-high glucose or galactose media, as described previously in [29]. Each individual providing a biological sample signed a written, informed consent approved by the Institutional Review Board of the Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy, in agreement with the Declaration of Helsinki.

2.8. MS/HPLC for Glutamate and Glutamine Quantification and Analysis of Amino Acids

10 heads from animals at 8 DAE were resuspended in 100 mL of Hepes/NaOH 50mM with proteases and phosphatases inhibitors (Roche, city, state abbrev if USA or Canada, country), samples were disrupted using a mechanical tip following two rounds of sonication of 10 s each. After centrifugation at 13,000 rpm for 30 min at 4 °C, 20 mL of each sample were set aside for Bradford analysis, while supernatants were diluted in buffer containing DL-Lysine D4 and DL-Ornithine D6 as internal standards (Cambridge Isotopes Labs, Inc. Eurisotop) and 2/3 *v/v* of methanol, to precipitate the proteins. After centrifugation, amino acid analysis was performed on a Perkin Elmer Series 200 HPLC equipped with a 4 mm, 2.0 × 75 mm Synergi Polar-RP (Phenomenex, city, state abbrev if USA or Canada, country) column, and coupled with triple quadrupole API2000 tandem mass spectrometer (AB SCIEX, city, MA, USA). AB SCIEX Analyst software (version 1.4, company, city, state abbrev if USA or Canada, country) was used for data acquisition and analysis. Analysis of free amino acids was performed by Innovhub, Stazioni Sperimentali per Industria,

Milan (Italy) with the following protocol: 30 heads of each genotype were resuspended in 300 mL of HPLC-grade H₂O, samples were disrupted using a mechanical tip following two rounds of sonication of 10 s each.

20 mL were set aside for Bradford analysis, while 75 mL of a 6% solution of sulfosalicylic acid were added to the final concentration of 1.5%. Samples were centrifuged for 20 min at 13,000 rpm, and supernatants were used for the analysis. The protocol applied (UNI 22615:1992) uses ionic exchange chromatography where the amino acids are separated by chromatography using an automatic Lithium High Performance Physiological Column with the instrument Biochrom 30+ from Biochrom. The separation is based on using Lithium Citrated buffer solutions employing a five steps pH separation from pH 2.80 up to pH 3.55. Amino acids were visualized on TLC using ninidrin and detected at 440 e 570 nm.

2.9. Quantitative Real Time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNAs were quantified using an ultraviolet (UV) spectrophotometer, and RNA integrity was confirmed with ethidium bromide staining. The purified RNA (1 mg) was used as the template for cDNA using SuperScript II (Invitrogen). The SYBR Green PCR Kit (Qiagen) was used, and products were quantified utilizing the BIO-RAD system. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) sample values were normalized for the expression of *actin-5C* mRNA. The relative level for each gene was calculated using the 2- $\Delta\Delta C_t$ method [30] and reported as arbitrary units. Three separate samples were used in triplicate. Primers were designed using Primer Express3 (Applied Biosystem). Primer's Sequences: *Gs1-F-TGCGTCTGCTGCGTACTGGC*, *R-CGGCGTTTCCAGGTTGCGGTA*; *actin5C-F-CACACCAAATCTTACAAAATGTGTGA*, *R-AATCCGGCCTTGACATG*.

2.10. Quantification of GS1 Activity

Drosophila larvae or heads that had been flash frozen in liquid nitrogen were resuspended in 200 mL 50 mM Hepes/NaOH buffer, pH 7.4 and 0.5% protease inhibitors cocktail (Sigma P8849). They were homogenized with a Branson 250 sonifier equipped with a microtip (25% power, 20% duty cycle) by applying four series of 10 sonication pulses and controlling the temperature by immersion in an ice bath. The homogenate was centrifuged in a microfuge at 13,000 rpm for 30 min at 4 °C and passed through an Ultrafree-MC (Millipore UFC306V) filter device by centrifuging at 11,000 rpm for 15 min at 4 °C. The filtrate was used for protein and activity assays. Protein concentration was determined with the Bradford method and bovine serum albumin as the reference protein. Glutamine Synthetase (GS) activity was assayed by quantifying the amount of γ -glutamylhydroxamate (GGHA) produced as described in [31–33]. In preliminary experiments a range of GGHA concentrations (0.07–0.15 mM) was used to determine the sensitivity and linear range of the assay. Briefly, a known amount of crude extract (0.1–0.5 mg in up to 120 mL) was incubated for 1 h at 37 °C in 50 mM Tris/HCl buffer, pH 7.5, hydroxylamine (40 mM final concentration), MgCl₂ (40 mM) L-glutamate (20 mM) and ATP (5 mM) in a final volume of 600 mL. The reaction was blocked by adding 200 mL of FeCl₃ solution (3.3% FeCl₃, 8% trichloroacetic acid (TCA), 0.67 M HCl) and centrifuged at top speed for 10 min. The amount of GGHA produced was calculated from the absorbance value at 500 nm and 600 nm with the HP 8453 diode array spectrophotometer using the reported extinction coefficients of 1.004 mM⁻¹cm⁻¹ and 0.471 mM⁻¹ cm⁻¹ [82] for the crude extracts derived from larvae and heads, respectively; by monitoring the entire spectrum of the final solution, we ensured that no artifacts arose from turbidity, precipitation or the presence of pigments, especially with *Drosophila* heads. For each assay a blank devoid of L-Glu and ATP was set up, and the measured absorbance was subtracted to that of the corresponding sample prior to the calculation of the activity value. The amount of larvae or heads extracts were selected in order to ensure that the GGHA concentration increase was linear with respect to the incubation time. L-Methionine sulfoxide (MSO) was added to reaction mixtures at a final concentration of 1 mM (from 10 mM stock solution in 50 mM Hepes/NaOH buffer, pH 7.4) to test its effect on GS1

activity. Activity was expressed as milliunits/mg protein, where 1 unit of GS activity is the amount of GS that converts 1 mmol L-Glu into product per minute.

GS activity detected in wild-type larvae was taken as 100% activity to normalize the values obtained with different larvae batches and in comparison with the values measured with transfected larvae. For each sample, replicates differed by less than 15%. GS1-specific activity in the heads of adult females was analyzed in the presence of the specific GS1-inhibitor methionine sulfoximine (MSO), as shown in Supplementary Figure S7.

2.11. Immunofluorescences and Quantification of the Size of Htt-Q93 Aggregates

To visualize human mutant Htt-Q93 aggregates, females *elav-Gal4*, *UAS-GFP* females were crossed with males carrying the appropriate UAS-transgenes. 3rd instar larvae were collected and washed in PBS. Brains were dissected and fixed for 30 min in 4% paraformaldehyde (PFA) on ice. After tissues permeabilization with 0.5% Triton X-100, samples were washed in PBS-0.1% Tween20 (PBST) and blocked in 3% BSA (in PBST) for 30 min on ice. Samples were incubated with anti-Htt antibody in 3% BSA overnight at 4 °C. After three washes of 10 min each in PBST, the samples were incubated with mouse or rabbit secondary antibodies Alexa 555 (Invitrogen) 1:400 in 3% BSA for 2 h and at room temperature (RT) on the shaker. After extensive washing with PBST, brains were mounted on slides in 20 mL of Mowiol. Nuclei were stained with Hoechst 33258 added at the final concentration of 1 µg/mL. The quantification of the mHtt aggregates visualized by fluorescence upon staining with anti-human Htt antibodies and secondary anti-rabbit Alexa 555, was performed on z-stacks acquired under a confocal microscope, by keeping the acquisition parameters constant. The intensity of the red aggregates was quantified from the confocal stacks using ImageJ 1.50i software (Wayne Rasband NIH, city, state abbreviation, USA) inside a square of fixed size (300 × 300 pixels), which was moved in four different regions in the specific area of the brain (calyx, VNC or IL). The aggregates were quantified by measuring the intensity of the fluorescent signal in these different regions (integrated density-IntDen tool on ImageJ). The integrated density of the fluorescence acquired was considered directly proportional to the aggregate number. Statistics was applied using as a threshold the IntDen in the control animals.

2.12. Statistical Analysis

Analysis of the variance was calculated using one-way ANOVA with Tukey multi-comparisons test. Student *t*-test analysis between genotypes was performed using GraphPad-PRISM7. One to four asterisks indicate: $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$, $p^{****} < 0.0001$, respectively.

2.13. Autophagy Analysis

elav-Gal4; *UAS-GFP*; *UAS-mCherry-Atg8a* females were crossed with males carrying the appropriate UAS-transgenes. Third instar larvae (72–80 h after hatching) were collected and washed, and then their brains were dissected in phosphate-buffered saline (PBS) buffer. Brains were fixed for 30 min in 4% paraformaldehyde (PFA) on ice. Nuclei were stained with Hoechst 33258, samples mounted in Mowiol and images acquired using a confocal microscope (SM5 LEICA Lesertechnik GmbH) using ImageJ 1.50i software or *AdobePhotoshop-CS4*. The quantification of autophagic puncta stained by mCherry-Atg8a and representing the autophagosomes, was performed on z-stacks acquired under a confocal microscope, by keeping the acquisition parameters constant. The intensity of the autophagic puncta was quantified with the software *ImageJ* inside a square of fixed size (300 × 300 pixels), which was moved in four different regions in the specific area of the brain (calyx, VNC or retina). Autophagy was quantified by measuring the intensity of the fluorescent signal in these different regions (integrated density-IntDen tool on ImageJ) using four out of five brains for each genotype of interest. Statistics was applied using as a threshold the IntDen in the control animals

3. Results

3.1. *Gs1* Rescues Neuronal Death Induced by Expression of Htt-Q93 in the Retina

In post mortem brains from HD patients, the expression of the enzyme glutamine synthetase (GS1) was shown to be reduced, similarly to our data in Western blot from human fibroblasts from patients with HD (see also Figure 7C). In order to evaluate the potential role of GS1 in this neurodegenerative disease we expressed *Drosophila Gs1*, homolog of the human *GLUL* gene (Supplementary Figure S1), in neurons of the retina using a well-established model for HD. These animals expressed *exon1*-of the human *huntingtin* gene containing 93 repetitions of the CAG sequence, herein called Htt-Q93 that recapitulate some phenotypes of the disease including neuronal loss and motility defects [34,35].

Ubiquitous expression of *Drosophila Gs1*, using the *EP-Gs1^{G3347}* line, resulted in a twofold increase in *Gs1-mRNA* (Figure 1A) and in a 50 kDa protein recognized by anti-human GS1 antibodies (Figure 1B). Analysis of GS1 enzymatic activity showed a significant increase compared to that in control *w¹¹¹⁸* animals (Figure 1C), suggesting that the *EP-Gs1G3347* line is expressing an active *Drosophila Gs1* enzyme.

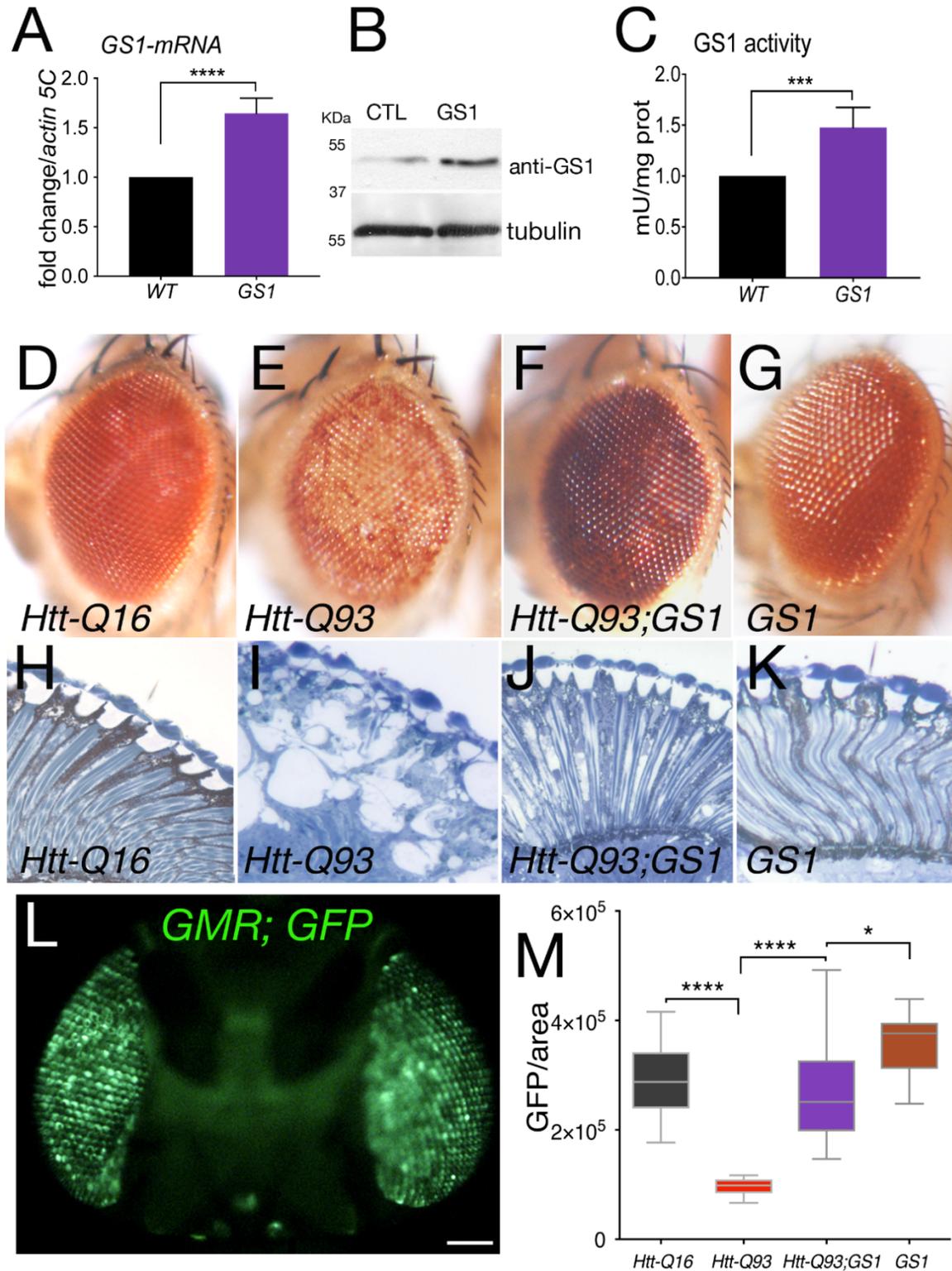


Figure 1. Expression of *GS1* in the retina rescues neuronal death induced by *Htt-Q93*. (A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *GS1* mRNA expression in larvae from control *w¹¹¹⁸* of animals expressing the *EP-GS1^{G4337}* (*GS1*) line using the *actin-Gal4* driver. The relative level of *GS1-mRNA* is reported as arbitrary units compared to *actin5C* used as a control. At least three separate experiments were performed in duplicate. (B) Western blot from larvae extracts showing the relative amount of *GS1* protein from *w¹¹¹⁸* (CTL) animals or expressing *GS1*. Tubulin was used as the loading control. (C) *GS1* enzymatic activity in extracts from whole larvae used in A and B. The *** = $p < 0.001$, **** = $p < 0.0001$ values in panels A and C were calculated from Student's *t*-test from at least three independent experiments.

(D–F) Photographs of *Drosophila*-compound eyes (lateral view) from females at 20 days after eclosion (DAE) expressing the indicated *UAS*-transgenes using the *GMR-Gal4* driver. (H–K) Representative photographs of the retina from animals of the indicated genotype. (L) Fluorescent image of a fly head expressing *UAS-GFP* under the *GMR-Gal4* promoter (sagittal view), bar 100 μ m. (M) Quantification of GFP from photographs of adult eyes at 20 DAE of the indicated genotypes. One-way analysis of variance (ANOVA) with Tukey multiple comparison for Figure 1M * = $p < 0.05$ and **** = $p < 0.0001$, and the error bars indicate the standard deviations. The whole ANOVA and data are in the supplementary Excel file.

When *UAS-GS1* was co-expressed with *UAS-Htt-Q93* in the retina using the *GMR-Gal4* promoter, we observed a complete rescue of the reduced pigmentation visible in the ommatidia of Htt-Q93 animals (compare Figure 1E with Figure 1F). On the contrary, reducing GS1 expression together with Htt-Q93 exacerbated these defects, and animals showed a premature loss of pigment cells and eye morphology defects, in that at 8 days after eclosion, it was similar to that of animals expressing *UAS-Htt-Q93* at 20 days (Supplementary Figure S2). An ultramicroscopic analysis showed that expression of GS1 ameliorated also the alteration of the cornea morphology induced by Htt-Q93 expression and visualized by the presence of large vacuoles indicating cell loss (Figure 1I,J). Morphological analysis of the retina from *UAS-GS1* and control *UAS-Htt-Q16* animals revealed no defects (Figure 1H,K, respectively).

Neuronal death can be indirectly analyzed by measuring the decrease over time of the fluorescence emitted by the Green Fluorescent Protein (GFP) when co-expressed with Htt-Q93 [35] (Figure 1L). These experiments showed that co-expression of GS1 completely rescued the reduction in GFP induced by the presence of Htt-Q93 (Figure 1M, ANOVA $p < 0.0001$), while we did not observe any significant difference in the quantification of GFP in the ommatidia of control *GMR-Q16* or *GMR-GS1* animals. A representative image for each genotype is shown in Supplementary Figure S3.

3.2. GS1 Expression in Neurons Improves Locomotion Defects of Htt-Q93 Animals

We next extended our analysis to all neurons using the *elav¹⁵⁵-Gal4* pan-neuronal promoter (hereon referred as *elav-Gal4*) [34]. Western blot analysis on extracts from larval brains from *elav-Htt-Q16* and *elav-Htt-Q93* animals showed the presence of a 35 kDa and 55 kDa proteins, respectively, recognized by anti-human HTT antibodies. In addition, a high-molecular-weight protein-complex > 250 kDa (HMW-mHtt) was visibly entrapped in the stacking gel, where extracts from *elav-Htt-Q93* animals were loaded, and almost no reduction in the HMW-mHtt was detected when *Htt-Q93* was co-expressed with *GS1* (Figure 2A), suggesting that GS1 was only partially able to reduce the high molecular weight form of HMW-mHtt aggregates at this stage of expression.

Since one characteristic of HD is the progressive impairment of movement [34], we investigated whether expression of *UAS-GS1* could rescue the motility defects in third instar larvae expressing *UAS-Htt-Q93* in neurons. These experiments showed that the expression of GS1 was able to fully rescue the motor defects that were already visible in Htt-Q93 larvae at 48–72 h after egg laying (AEL) (Figure 2B, ANOVA $p < 0.0001$ and Supplementary Figure S4). Expression of *UAS-GS1* also rescued the developmental lethality of Htt-Q93 animals (Figure 2C, ANOVA $p < 0.05$). No defects in morphology or motility were observed in larvae expressing *UAS-GS1* or *UAS-Htt-Q16*. Similarly, Western blot analysis in the heads from adult females showed a pattern of expression for Htt-Q16 and Htt-Q93 proteins similar to that in the larval brains (Figure 2D), and in addition, a similar level for GS1 protein was shown in extracts from the heads of animals expressing *UAS-Htt-Q93* or *UAS-GS1* (Figure 1E). GS1 enzymatic activity increased in protein extracts from heads of animals expressing *UAS-GS1* (Figure 2F, ANOVA $p < 0.05$), and it was similar in extracts from animals expressing *UAS-GS1* alone or together with *UAS-Htt-Q93* (Figure 2F, ANOVA $p = ns$). Finally, climbing activity, assayed on adult females, showed that the co-expression of GS1 ameliorates the motility defects of *elav/+; Htt-Q93* animals that presented a 50% reduction in their motility at 8 days (Figure 2G,L, ANOVA $p < 0.0001$), while the expression of GS1 extended their

50% of success up to 14 days (Figure 2G, ANOVA $p < 0.05$). A similar effect was observed for the ability of GS1 to promote survival (Figure 2H, ANOVA $p < 0.05$ and panel L).

Flies expressing GS1 alone or Htt-Q16 did not show any visible alterations in their motility or viability. We then analyzed the ability of GS1 to rescue neuronal death by quantifying the expression of the neural proteins Elav and Synapsin (Figure 2I). These data indicate that, while expression of Htt-Q93 significantly decreased the level of Elav (69%) and of Synapsin (38%), the presence of GS1 completely rescued Elav expression and partially Synapsin (90%), corroborating our data on its function on neuronal survival.

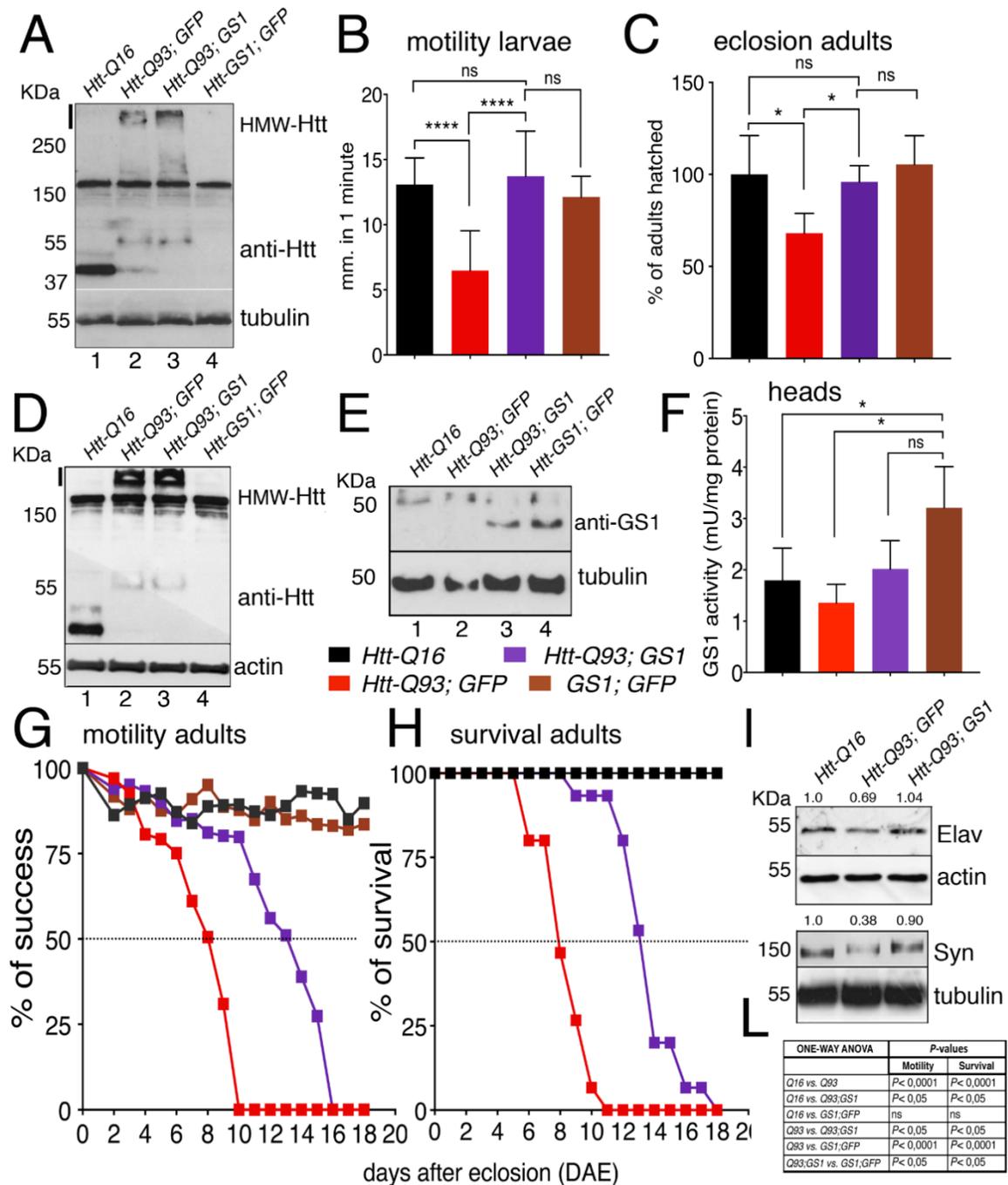


Figure 2. Expression of GS1 in neurons rescues motility defects and neuronal death induced by Htt-Q93. (A) Western blot showing the relative amounts of the protein huntingtin in lysates from 20 third instar larval brains expressing the indicated transgenes using the pan-neuronal driver

elav-Gal4. Tubulin was used as the loading control. The band of about 30 KDa in lane 1 represents the Htt-Q16 protein, while the band at 55 KDa, in lanes 2 and 3, represents the Htt-Q93 protein. The high molecular weight proteins recognized by the anti-hHTT antibody also present in lanes 2 and 3 represent the mutant Htt high form that jammed in the stacking gel (indicated by a black line on the side). The 150 KDa band present in all the lanes is aspecific, since it is recognized by the mHtt antibody also in lane 4 where Htt is not expressed. A similar description is applied to the Western in Figure 2D. (B) Quantification of the motility in third instar larvae of the indicated genotype, where the data are expressed as the distance traveled in one minute measured in mm, and 10 larvae from each genotype were used the experiment was repeated three times (see Supplementary Figures S4C–E, movies S1 and S2). (C) Eclosion timing of adult females of the relative genotypes. (D,E) Western blots showing the respective amounts of huntingtin and GS1 proteins in lysates from 15 heads for each genotype, of females at 8 days after eclosion (DAE), wherein actin and tubulin were used as a loading control, respectively. In 2E is visible a band of about 30–40 KDa only when the *UAS-GS1* transgene is expressed (lane 3 and 4). The band at the higher molecular weight of about 55 KDa is either the endogenous *Drosophila* GS1 that is recognized by the anti-human GS1 antibody, or an aspecific band, since it is present in all the lanes also where GS1 is not overexpressed. (F) GS1 enzymatic activity (mUnits/mg of total proteins) in lysates from heads of females at 8 DAE, data show the significant increase of GS1 activity (brown) in these animals compared to that in the heads of un-transfected control Q16 animals (black bar), and from Htt-Q93 (red) or Htt-Q93 co-expressing GS1 (purple). The $* = p < 0.05$ values in F were calculated from Student's *t*-test from at least three independent experiments, and error bars indicate the standard deviations. (G) Motility assay on adult females, where data are expressed as % of success (see Supplementary Figure S4F, movie S3). (H) Analysis of adult lethality is expressed as the % of survival of females relative to control. The ability of GS1 to ameliorate the motility of *Htt-Q93* was confirmed using three independent *Htt-Q93*, *GS1* recombinant lines (Supplementary Figure S8). (I) Western blot showing the amounts of endogenous neuronal specific proteins elav and synapsin in lysates from fifteen heads of the indicated genotype, where actin and tubulin were used as control, and the % of reduction of the protein is calculated compared to control that is considered equal to 100%. (L) Table reporting ANOVA for the data in panels G and H. The $**** = p < 0.0001$ and $* = p < 0.05$ values in panels B, C and F were calculated using one-way ANOVA with Tukey multiple comparison from at least three independent experiments, and error bars indicate the standard deviations. The whole ANOVA and data are in the supplementary Excel file.

3.3. Expression of GS1 Reduces Htt-Q93 Protein Aggregates in Neurons

Mutant huntingtin protein forms toxic intracellular aggregates that largely contribute to neuronal death. Using the *elav-Gal4* promoter, that is transcriptionally active in neurons and in the gigantic cells of the salivary glands, we expressed *UAS-Htt-Q93* together with membrane *UAS-GFP* and used an anti-human Htt antibody to detect the presence of Htt-Q93 protein aggregates (mHtt) by immunofluorescence (Figure 3A–D,F,G).

At 72 h of development, mHtt-aggregates were clearly visible in the cytoplasm and in the nuclei of the cells of the salivary gland (SG) (Figure 3A) and in the cells of the calyx of Htt-Q93 animals (Figure 3B), but absent in cells of the calyx from Htt-Q16 larvae (Figure 3C). The ability of GS1 to modify the formation of Htt Q93 aggregates was analyzed by quantifying the immunofluorescence in neurons from the Intra-Lobe (IL), and from the Ventral Nerve Cord (VNC) (Figure 3F). This analysis was performed using the signal for the integrated density of the fluorescence acquired from z-stacks using a defined threshold that considers the number and size of the aggregates (collaboration with the Imaging Facility at CIBIO). This analysis showed that co-expression of *GS1* with *Htt-Q93* significantly reduced the number of mHtt aggregates, in neurons from the IL (Figure 3G,H, *t*-student, $p < 0.01$) and from the VNC (Figure 3I, *t*-student, $p < 0.01$). To support these results we performed a filter-trap assay, a technique that is used to trap large protein aggregates on a cellulose acetate membrane where they can be visualized by immunoblot. These experiments showed that the co-expression of *GS1* reduced the amount of large Htt-Q93 aggregates trapped on the membrane, while no Htt-Q93 aggregates were detected in

extracts from the brains of animals expressing *UAS-Htt-Q16* or *UAS-GS1* alone (Figure 3J). Similar experiments were performed in adult animals using extracts from females' heads at 10 DAE.

These experiments confirmed that also in adult animals the expression of GS1 significantly reduced the presence of Htt-Q93 aggregates (Figure 3M, integrated analysis in analysis 3J, *t*-student, $p < 0.001$), suggesting that GS1 activity in neurons induces a mechanism that affects the formation of the toxic Htt-Q93 protein aggregates that is conserved after development.

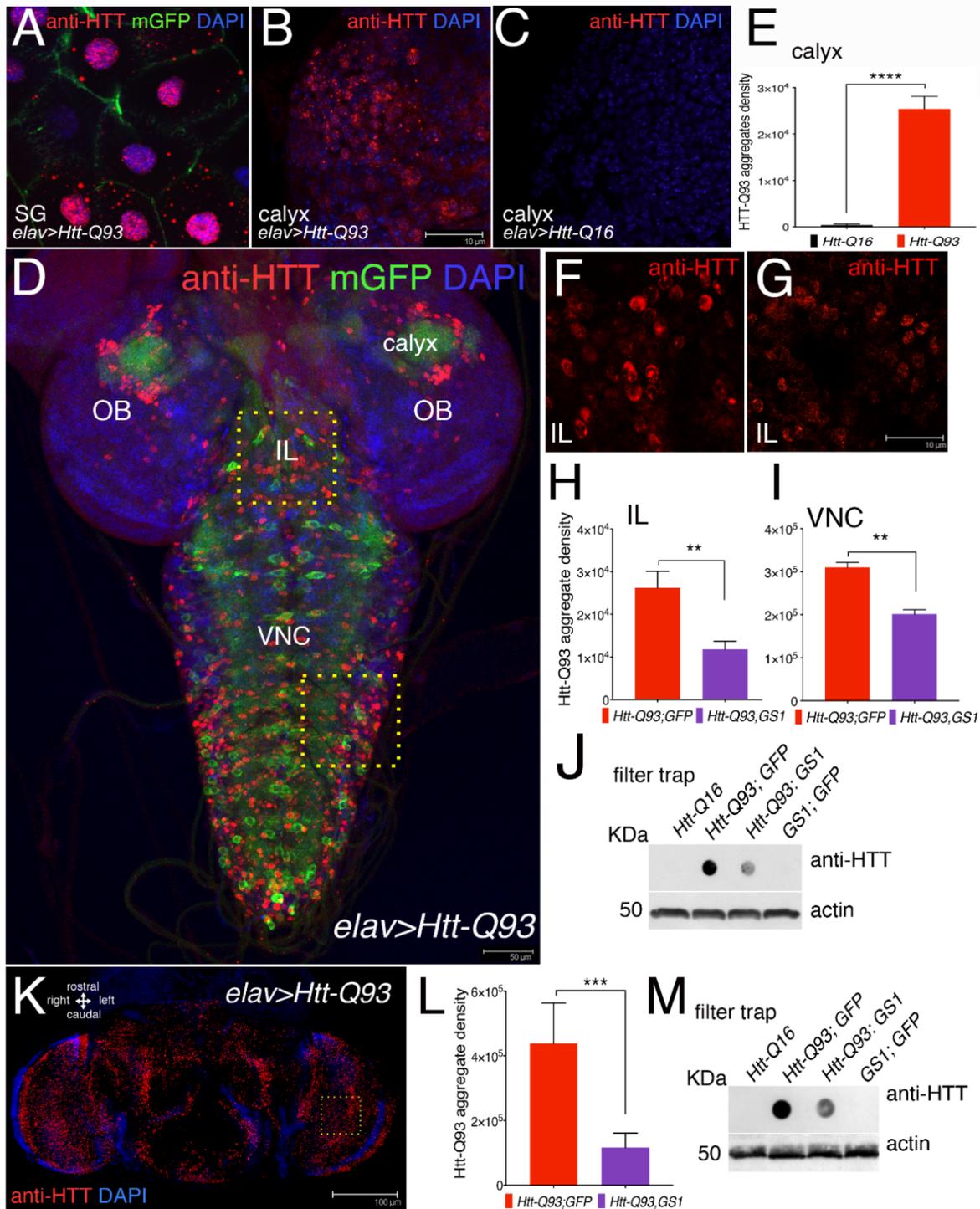


Figure 3. Expression of GS1 in neurons reduces the presence of Htt-Q93 aggregates. (A) Confocal microscopy images of cells from the salivary gland (SG), where the *elav-Gal4* insertion is also transcriptionally active, showing the presence of Htt-Q93 protein aggregates (red), that are visible in

the cytoplasm and in the nuclei of cells expressing *UAS-Htt-Q93* and *UAS-GFP*, immunostained with anti-hHtt antibodies. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI).

(B) Htt-Q93 aggregates immunostained in the region from the calyx, (C) that are not detected in the calyx from control *Htt-Q16* animals; bar 10 μm ; (E) relative quantification of the fluorescence in (B,C). (D) Confocal image of the whole brain from third instar larvae expressing Htt-Q93 and GFP (*elav-Gal4; Htt-Q93; mGFP*); the Htt-Q93 aggregates are visualized by immunofluorescence and nuclei with DAPI, bar 50 μm . The yellow squares represent the regions where the Htt-Q93 aggregates were quantified: the Ventral Nerve Cord (VNC) and the intralobe region (IL), in addition to the calyx. (F,G) Picture representing the Htt-Q93 aggregates visualized in the intralobe (IL) region from *Htt-Q93* (F) or *Htt-Q93; GS1* (G) animals; bar 10 μm ; the relative quantification analysis is shown from the IL (H) and in the Ventral Nerve Cord (VNC) (I). Quantification of the aggregate density was calculated in the relative area and measured as the integrated density of the immunofluorescence signal visualized using anti-human Htt antibody from 10 confocal z-stacks for each animal and elaborated with ImageJ, (see Materials and Methods). (J) Filter trap analysis using lysates from the brains of the indicated transgenes; the expression of actin in the same extracts was analyzed by western blot as an internal control for loading. (K) Confocal image of *elav-Gal4; Htt-Q93* adult female's head at 10 DAE, immunostained with anti-hHtt antibody showing the presence of Htt-Q93 aggregates (RED, nuclei DAPI), bar 100 μm . (L) Quantification of the Htt-Q93 aggregates in the heads of the animals of the indicated genotypes from confocal z-stacks; analysis was performed in the area indicated by the square. (M) Filter trap analysis of Htt-Q93 protein aggregates using lysates from the heads. Actin was used as the internal control. The $** = p < 0.01$, $*** = p < 0.001$ values in panels E, I, J and M were calculated from Student's *t*-test from at least three independent experiments, where error bars indicate the standard deviations. OL = optic lobe.

3.4. GS1 Induces Autophagy in Neurons

Neurons employ autophagy to efficiently clear cellular debris that are detrimental for their homeostasis, including toxic protein aggregates [36]. To investigate whether GS1 may activate the autophagy-lysosomal pathway, we analyzed whether or not its expression induces the formation of the characteristic autophagosome vesicles, visible as autophagic puncta and marked by the ubiquitin-like protein Atg8a, the homolog of the human LC3, that localize at the autophagosome [37]. We used Atg8a fused with the mCherry-reporter [38] that was co-expressed with mGFP in neurons using the *elav-Gal4* promoter. Fluorescence associated to mCherry-Atg8a was analyzed in the third instar larvae, specifically in the neurons of the calyx, the Ventral Neuronal Cord (VNC) and the retina (Re) (Figure 4A).

This analysis showed that the expression of *UAS-GS1* alone was able to induce the formation of autophagosomes, in addition its expression significantly rescued the reduction in the number of autophagic puncta induced by *UAS-HttQ93* in all the regions analyzed (Figure 4H, in the calyx, ANOVA $p < 0.0001$; 4K in the VNC, ANOVA $p < 0.05$ and 4L in the retina, ANOVA $p < 0.05$). A similar analysis was performed in the adult animals, where the integrated density of the autophagic puncta was quantified in the female brains at 10 DAE (Figure 4M). These data are consistent with those from larval brains, and show that the co-expression of GS1 alone induces the formation of autophagosomes, while with Htt-Q93, it results in a significant rescue of the autophagic puncta (Figure 4M; ANOVA $p < 0.01$), and no significant differences were found in the number of autophagosomes between *UAS-Htt-Q93, GS1* and *UAS-GS1* animals (ANOVA $p = \text{ns}$).

We then analyzed genetically whether the ability of GS1 to ameliorate the motility in Htt-Q93 animals was dependent upon a functional autophagic pathway. Using the RNAi technique, we reduced the expression of Atg1/ULK1 or Atg5, key components of the macroautophagy pathway [39] in combination with the expression of GS1. These data showed that the ability of GS1 to ameliorate the climbing defects of Htt-Q93 animals was significantly lost when Atg1 or Atg5 expression was reduced (Figure 5A, *Htt-Q93, GS1* vs., *Htt-Q93, GFP; Atg1-RNAi* ANOVA $p < 0.0001$; and *Htt-Q93, GS1* vs., *Htt-Q93, GFP; Atg5-RNAi* ANOVA $p < 0.0001$), suggesting that the rescue exerted by GS1 requires a functional autophagic pathway.

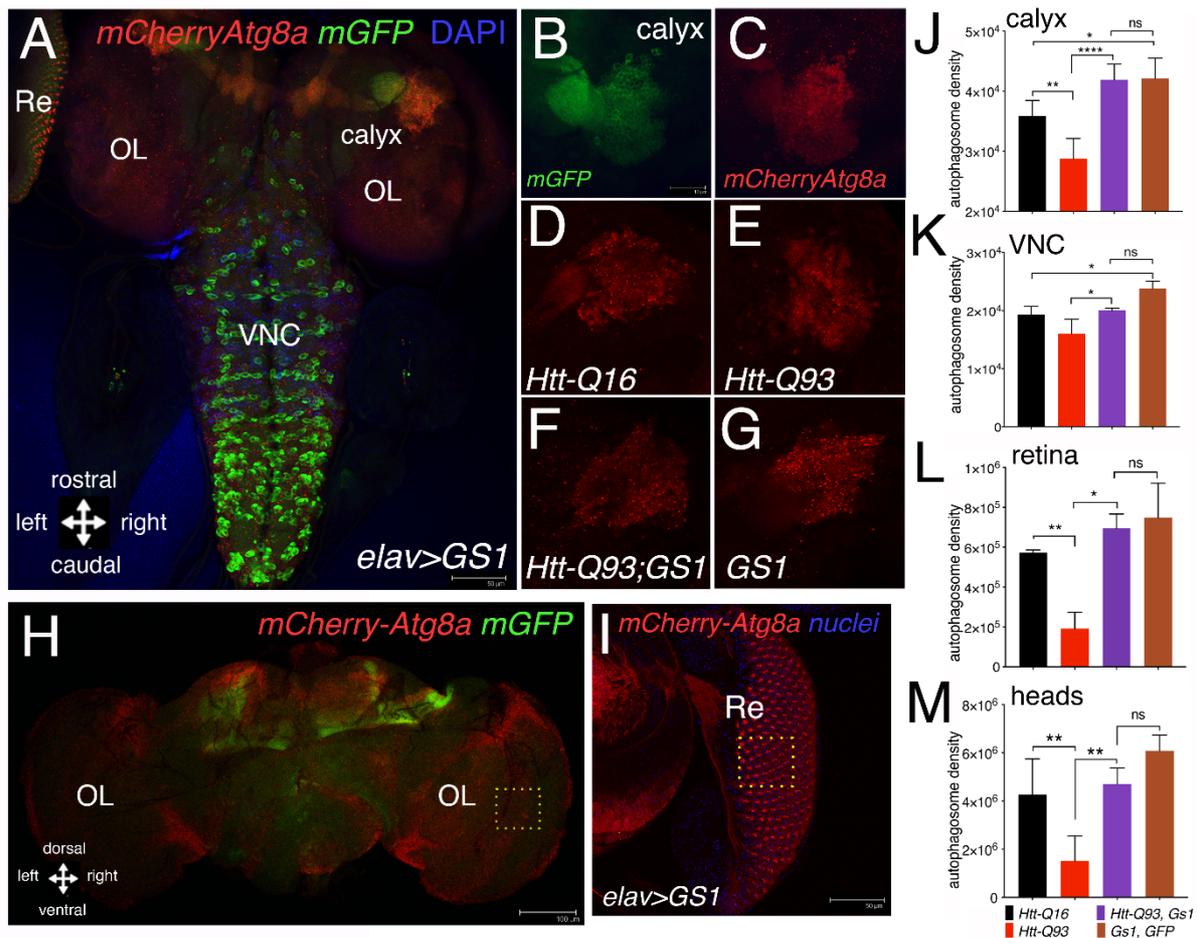


Figure 4. Expression of GS1 in neurons induces autophagy. **(A)** Confocal image of whole the brain from the 3rd instar larva showing the pattern of expression for *elav-mCherryAtg8a*; *GFP*; neurons are marked by the *GFP*; bar 50 μ m. The images represent the pattern of expression of *elav-mCherryAtg8a* in neurons from animals expressing *GS1* (images for control *w¹¹¹⁸* and *GS1* animals are shown in Supplementary Figure S10). Autophagic puncta were analyzed in the region of the calyx, retina (Re) and in the Ventral Nerve Cord (VNC). OL = optic lobe. **(B,C)** Insets showing a higher magnification of the calyx; neurons are visualized by *elav-GFP* **(B)** and autophagic puncta by *elav-mCherryAtg8a* **(C)**; bar 10 μ m. **(D–G)** representative images showing *mCherryAtg8a* staining in the calyx of the animals at the indicated genotypes, (a higher magnification of these images is shown in Supplementary Figure S10). **(H)** Image of an *elav-mCherry-Atg8a*; *mGFP* female's head at 10 DAE; OL = optic lobe; bar 100 μ m. **(I)** Image of an eye disc from a third instar *elav-GS1* larva co-expressing *mCherry-Atg8a* in neurons of the retina and in the pigmented cells, with the nuclei stained in blue; bar 50 μ m. **(J–M)** Quantification of autophagy was measured as the integrated density of *mCherry-Atg8a* and then converted in autophagosome density (see methods). Autophagosome density in neurons of the calyx, **(J)** in the Ventral Nerve Cord (VNC), **(K)** in the retina **(L)** and in the adult heads **(M)** from animals of the indicated genotypes. One-way ANOVA *p*-values analysis: **** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$ were calculated from a minimum of five animals, error bars indicate the standard deviations. ANOVA *p*-values for these experiments are in the supplementary Excel file.

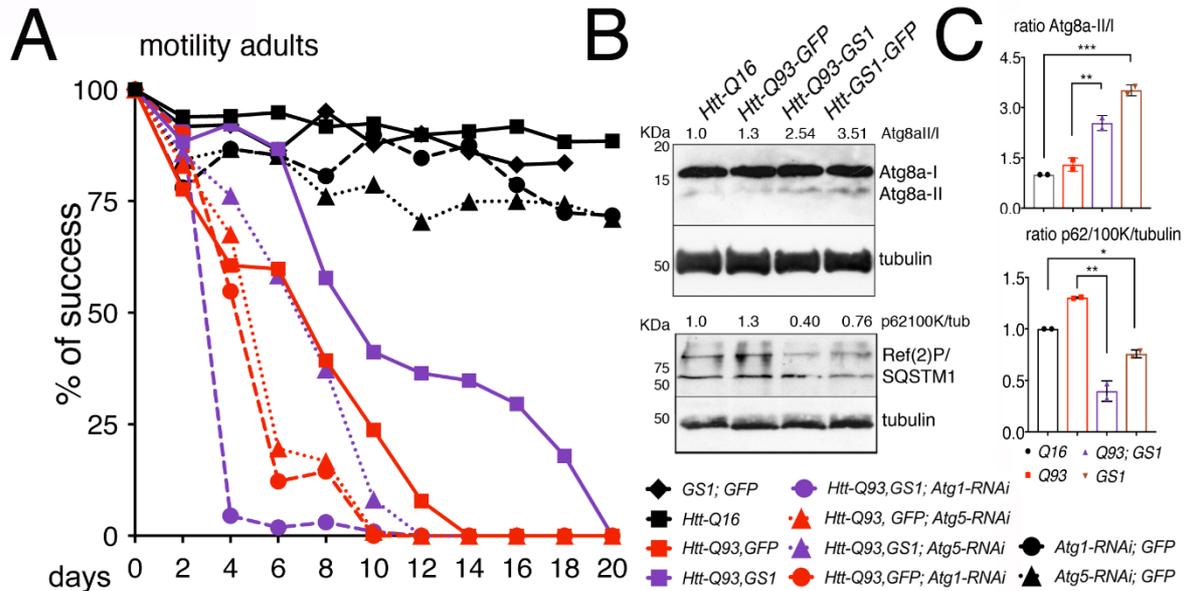


Figure 5. Reduction of *Atg1* and *Atg5* significantly impairs the ability of GS1 to rescue the motility defects induced by *Htt-Q93*. **(A)** Motility assays, the *p*-values from the one-way ANOVA in graph A is reported in the supplementary Excel file. 15 animals were used for each genotype. The efficiency of the *Atg1* and *Atg5 RNAi* lines was tested for their ability to reduce autophagic puncta and in Western blot experiments (Supplementary Figure S9). **(B)** The presence of GS1 favors Atg8a cleavage and reduces the presence of Ref(2)P. Western blots using cell extracts from 10 heads of females at 10 DAE of the indicated genotype, showing the pattern of expression of Atg8a-I and II (using anti GABARAP antibodies) and of SQSTM1/Ref(2)P, note that in *Drosophila* the latter runs in two isoforms of about 100 and 62 KDa, both recognized by the *Drosophila* antibody. Tubulin was used as the control for loading. Experiments were repeated at least twice with similar results. **(C)** The ratio for Atg8a-II/I and Ref(2)P/tubulin were calculated by comparing the quantification of the relative proteins to that in control *Htt-Q16* animals, previously normalized to tubulin with ImageJ. We calculate the ratio using the 100 KDa isoform, since is described as the ortholog of human p62 [40]. One-way ANOVA *p*-values: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ were calculated from two independent experiments of animals, and error bars indicate the standard deviations. ANOVA *p*-values for these experiments are in the supplementary Excel file.

In autophagy, the maturation of the phagosome determines the amount of LC3 (Atg8a in *Drosophila*), that is recruited to the vesicles, and this depends on the p62/SQSTM1 (sequestosome1) (Ref(2)P in *Drosophila*) necessary for the recruitment of the ubiquitinated substrates to the autophagosomes [40,41]. The expression levels of Atg8a and Ref(2)P proteins are used in Western blot to mark a functional autophagic flux, where the Atg8a-I protein (15 kDa) is lipidated and cleaved to yield a fast-migrating band of approximately 7 kDa (Atg8a-II), while its direct interaction with Ref(2)P, by virtue of its ubiquitin-associated domain, results in Ref(2)P degradation. Our analysis showed that THE expression of GS1 increased the ratio of Atg8a cleavage as the amount of the fast-migrating Atg8a-II band increased upon GS1 expression (Figures 5B,C) and was visible also when GS1 was co-expressed with Htt-Q93 (Figure 5B,C). Analysis of SQSTM1/Ref(2)P expression showed two distinct bands of approximately 100 and 62 KDa recognized by the antibody, that were reduced in the presence of GS1 (Figure 5B,C), and when GS1 was co-expressed with Htt-Q93 (Figure 5B,C). Even though these changes are not strong, they are significant (Figure C), and also on an average of two experiments, suggesting that GS1 promotes in neurons a small but active autophagic flux.

3.5. Expression of GS1 Reduces TOR Activation Visible by Phosphorylation of Its Substrate S6K

When it is activated, TOR translocates onto the lysosomal membrane where it assembles in the TORC1 complex, resulting in its activation and phosphorylation of the downstream targets S6K and 4EBP [42]. To explore the ability of the GS1-affected TOR activation, we analyzed the level of phosphorylation of S6K on Thr-398, as it is a known target of TOR kinase, and it is conserved also in *Drosophila* S6K [43]. This analysis revealed that the expression of *UAS-GS1* in neurons significantly reduced the S6K phosphorylation detected on the whole head lysates (Figure 6A,B), and a similar result was obtained when *UAS-GS1* was co-expressed with *UAS-Htt-Q93* (Figure 6A,B). These data suggest that GS1 activity is able to reduce the level of essential amino acids, resulting in the inhibition of TOR complex1 and of its downstream pathway.

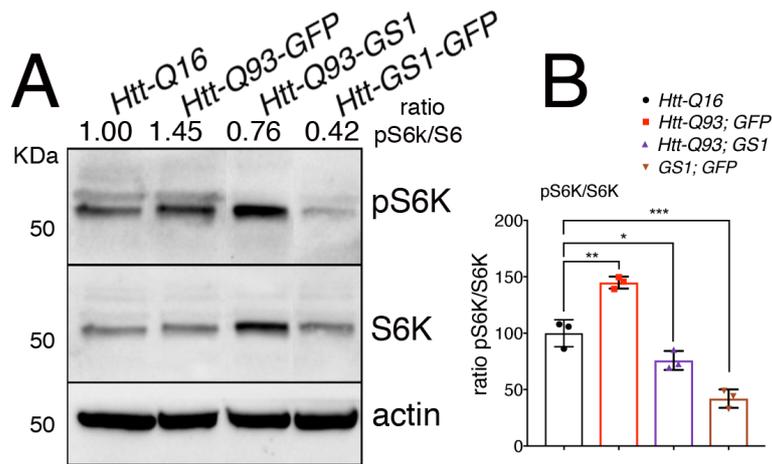


Figure 6. Expression of GS1 in neurons reduces S6K phosphorylation. (A) Western blot showing the level of phosphorylated S6K on Thr 398 and of S6K in lysates within 10 heads from animals expressing the indicated transgenes in neurons using the *elav-Gal4* driver. Actin was used as the loading control. (B) Graphic representing the ratio of S6K phosphorylation versus the unphosphorylated form of the protein. The ratio between pS6K/S6K was calculated from three independent experiments. One-way ANOVA *p*-values were calculated from a minimum of three independent experiments, and the error bars indicate the standard deviations. ANOVA *p*-values for the experiment are in the supplementary Excel file. **** = $p < 0.0001$, ** = $p < 0.001$.

3.6. Expression of GS1 in Neurons Reduces Some Essential Amino Acids

We found that glutamate levels increased in the head of animals expressing Htt-Q93 in neurons (Figure 7A, solid black vs. solid red columns, *t*-test $p < 0.0001$), and these levels are significantly reduced by the expression of GS1 (Figure 7A solid black vs. purple columns, *t*-test $p < 0.001$), an effect that is accompanied by a significant increase in glutamine level (Figure 7A, empty black vs. empty red columns, *t*-test $p < 0.01$), indicating that GS1 was able to convert glutamate into glutamine *in vivo*.

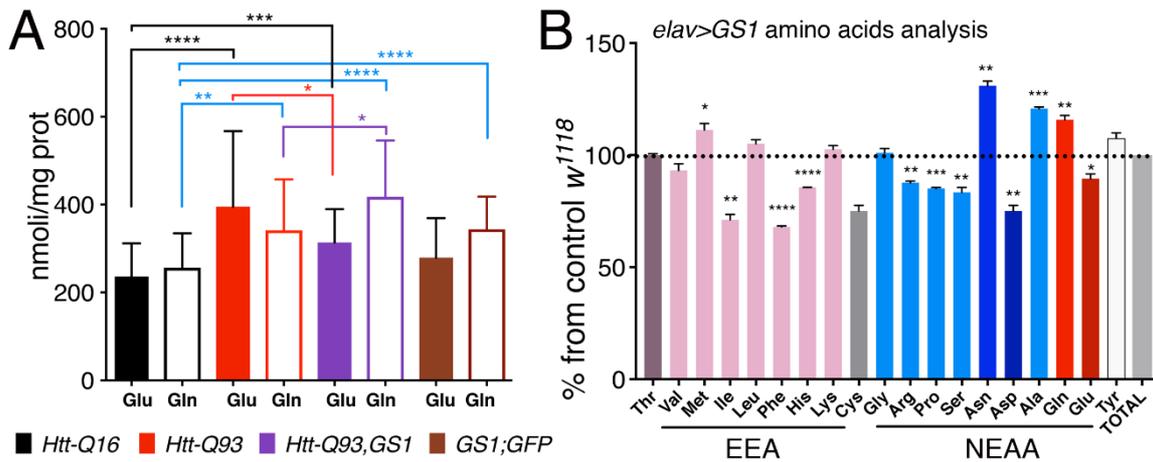


Figure 7. Expression of *GS1* in neurons reduced the Glu and increases Gln, and influences amino acid levels. **(A)** Quantification of glutamate (Glu) and glutamine (Gln) (MS/HPLC) in lysates from 20 heads of animals of the indicated genotypes. **(B)** Amino acids profiling from 20 heads of animals expressing *GS1* in neurons, and the data are indicated as the % of the AA concentrations in *GS1* versus the relative concentration in lysates from control *w¹¹¹⁸* animals. EAA = essential amino acids, NEAA = non-essential amino acids. The **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ values were calculated from Student's *t*-test from at least three independent experiments, and the error bars indicate the standard deviations.

Notably, *GS1* expression in vivo was able to reduce the level of glutamate also when co-expressed with Htt-Q93 (Figure 7A, solid red vs. solid purple columns, *t*-test $p < 0.05$), and to significantly increase glutamine levels (Figure 7A, empty red vs. empty purple columns, *t*-test $p < 0.05$). To identify significant changes induced by *GS1* in the pattern of all amino acids, we analyzed the profile of Essential amino acids (EAA) and Non-Essential amino acids (NEAA) from the heads of animals expressing *GS1* in neurons, and compared it to that from control Htt-Q16 flies (Figure 7B). Our data confirmed that glutamine (Gln) was significantly increased upon *GS1* expression ($p < 0.01$) and the glutamate level was reduced ($p < 0.05$). In addition, EAA:isoleucine (Ile), phenylalanine (Phe), histidine (His) and NEAA:arginine (Arg), proline (Pro), serine (Ser) and aspartic acid (Asp) were significantly reduced. In addition, these data revealed the ability of *GS1* to increase asparagine (Asn) while reducing aspartic acid (Asp). This last observation, together with the increase of the glutamine level, may suggest that the expression of *GS1* in neurons activates asparagine synthetase (ASNS), an enzyme that hydrolyzes glutamine to produce asparagine from aspartic acid and glutamate. Notably, this enzyme was described as activated to bypass amino acid starvation in mammals, for the production of Asp necessary to sustain protein synthesis [23].

3.7. Fibroblasts from Patients with Huntington's Disease have Impaired Autophagy and Reduced Level of *GS1*

Since autophagy is known to be defective in HD [44,45], we analyzed whether the autophagic flux was compromised in fibroblasts derived from patients carrying different lengths of the (CAG)s in the *exon1*-of the *Huntingtin* gene (*Htt*). Our characterization of hHtt protein expression in these cells showed the presence by Western blot of a band running above 250 kDa and possibly the full-length Htt protein with the predicted size of 350 kDa [46]. In addition, we observed a band of about 70 kDa only present in lysates from cells derived from HD patients carrying an average of 78–90 CAG repeats, while a band of about 55 kDa was present in all the samples, but more evident in lysates from fibroblasts derived from healthy donors carrying a sequence of about 17–33 CAG repeats. Since hHtt protein is known to be cleaved by calpain at around amino acids 437–586 to generate a series of proteolytic fragments of about 50 kDa [47], we believe that the difference in the molecular weight observed in the lysates from HD (mHtt, about 70 kDa), compared to healthy donors (wt-Htt, below 50 kDa), is the result of the presence of a different length in the CAGs

sequence responsible for the difference in the molecular weight of the different proteolytic fragment and recognized by the anti-*N*-terminus Htt antibodies.

The proteolytic cleavage of LC3 is often considered a hallmark of autophagy, and our analysis on the level of expression of LC3-II showed that in cells from HD patients, the fast-migrating bands, corresponding to the cleaved LC3-II, were significantly reduced (Student *t*-test $p=0.0341$), suggesting that somehow the autophagic flux has been compromised in these cells (Figures 8B,C). In addition, we analyzed the level of GS1 protein expression, and to our surprise, we found that GS1 protein was 45% reduced in fibroblasts from HD patients, as compared to that in fibroblasts from control donors (Figures 8D,E, Student *t*-test $p = 0.0132$). Taken together, these data in humans corroborate our results described in flies that suggest a potential novel conserved function for GS1 to control amino acid metabolism and autophagy, a function that may be impaired in pathological conditions such as HD.

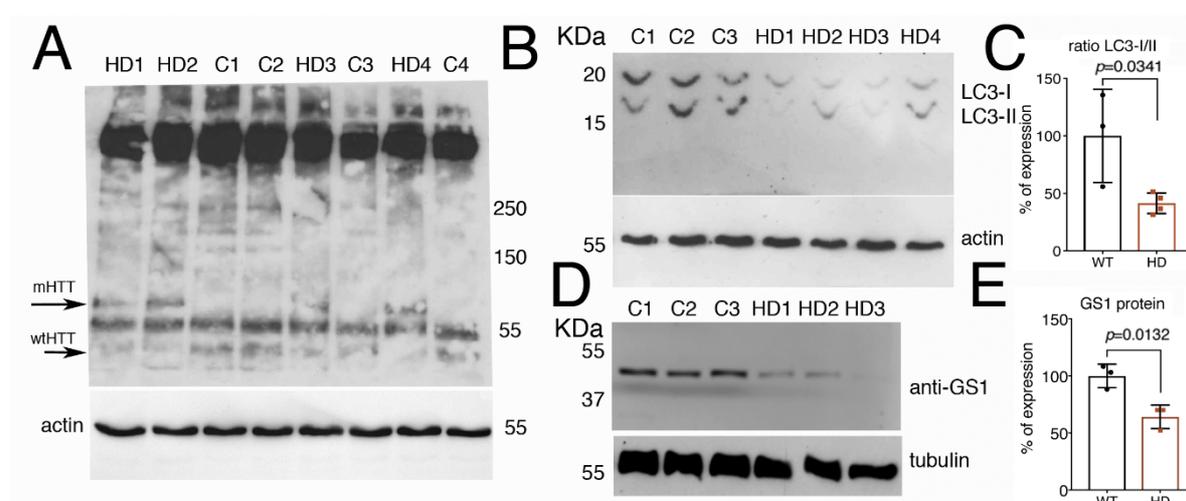


Figure 8. Fibroblasts from HD patients have reduced the expression of LC3 and GS1 proteins. (A) Western blot showing the pattern of expression of human Htt protein in fibroblasts from control donors (C1–C3), with an average of 15–33 CAGs, or from HD patients (HD1–HD4) with an average of 78–90 CAGs. The anti-hHtt antibodies evidence the presence of a band of about 70 kDa (mHTT) that is visible only in lysates from cells deriving from HD patients, while in the lysates from controls, a band below 55 kDa is evident and preferentially expressed in healthy donors (wt-Htt). Experiments were repeated at least twice, data were analyzed from three different patients or controls, and a representative situation is shown. (B) Western blot showing the cleavage of LC3 protein in the lysates of fibroblasts from control donors and from HD patients. The fast-migrating forms of LC3-II (upper panel) are significantly reduced in the extracts from fibroblasts of HD patient as compared to lysates from control donors. Actin is shown as a control for loading (lower panel). (C) Quantification of the ratio LC3-II/I from figure B. (D) Western blot showing that expression of GS1 in fibroblasts from HD patients is reduced compared to its expression in control donors, where tubulin was used as a control for loading. (E) relative quantification analysis of GS1 in fibroblasts. The *p* values indicated were calculated from Student's *t*-test from the relative number of samples.

4. Discussion

The glutamate–glutamine cycle (GGC) is a sequence of biochemical events between neurons and glia necessary for the control of glutamate homeostasis. Previous studies showed that this cycle is impaired in mouse and *Drosophila* models for HD [48,49]. In addition, the activity of Glutamine Synthetase-1 (GS1), a key regulator of the GGC, was found to have been reduced in post mortem brains of HD patients [50,51]. To elucidate the role of GS1 in HD, we modulated its activity in neurons using a *Drosophila* model expressing the mutated human exon1 *Htt-Q93* that mimics many aspects of the neurodegeneration induced by toxic polyglutamine expansion in HD patients [34].

Our data show that neuronal expression of the *Drosophila* homolog of human GS1 ameliorates animal motility and reduces neuronal death induced by the expression of *Htt-Q93*.

Moreover, we found that co-expressing GS1 with *Htt-Q93* reduces the size of the toxic Htt-Q93 aggregates, and increases the levels of macro-autophagy, herein referred to as autophagy, establishing a novel link between the GGC and functional autophagy in post-mitotic cells.

Autophagy has a well-established beneficial role in several processes relevant for neurons under both normal and pathological conditions [6,52]. It is a conserved mechanism controlled by key proteins like Atg1/ULK1, a kinase essential for the initial formation of the complex, then Atg5, that is involved together with LC3/Atg8 in the autophagosome maturation and lipidation of LC3/Atg8 to its proteolytic cleavage to Atg8-II. p62/SQSTM1/Ref(2)P binds to the autophagosome-coating protein LC3/Atg8 and activates the fusion of the phagosome with the lysosome to degrade its cargo [21]. Polymorphisms in the *ATG* genes, including *ATG5* and *ATG7*, have been associated with an early onset of neurodegenerative diseases, including HD [53]. Moreover, the important role of autophagy in neurons was revealed by the conditional knock-out of *ATG5* and *ATG7* that results in progressive motoneuron defects, alteration in autophagosome formation and accumulation of ubiquitin-positive protein aggregates in degenerating neurons [54–56]. These phenotypes are conserved in *Drosophila*, where *atg7^{D77}* mutants have a short life-span, reduced motility and the accumulation of ubiquitinated proteins [57].

Our data show that the ability of GS1 to ameliorate the motility of *Htt-Q93* flies depends on a functional autophagic pathway, since the reduction of *atg1* and *atg5* completely abolished the rescue by GS1. Moreover, the presence of GS1 in neurons enhances the cleavage/lipidation of Atg8 (Atg8-II) and reduces the level of Ref(2)P/SQSTM1, suggesting an increase of the autophagic flux in neurons. Autophagy is inhibited by nutrients and amino acids that induce TORC1 activation [10,21]. Stimulation of TORC1 at the lysosome membrane depends on the ability of amino acids to activate the Rag(s) (GTPases), the Ragulator complex and the vacuolar H⁺ adenosine triphosphatase (v-ATPase) [58]. However, TOR activity is controlled specifically by certain branches of amino acids [59], most relevantly, glutamine [60,61], leucine [42,60,62], and arginine [63].

Our data show that expression of GS1 reduces the phosphorylation of the S6K protein, a target of TOR, thereby suggesting that it plays a role in the regulation of its activity. GS1 converts glutamate into glutamine, and our amino acids analysis shows that GS1 increases the level of glutamine alone and in the presence of *Htt-Q93* (Figure 7). Glutamine (Gln) is involved in a multiplicity of metabolic pathways, and its function in the activation of TOR still presents some controversial results. Gln in epithelial cells binds to the adenosine diphosphate ribosylation factor-1 GTPase (Arf1) to directly activate TORC1 at the lysosome membrane [59]. Our data in neurons however show that in the presence of GS1, TOR signaling is reduced even if the glutamine level increases, so this may suggest that in terminally-differentiated cells, GS1 induces a “starvation-like” condition that favors autophagy without the activation of TORC1. In support of this mechanism, van der Vos and colleagues showed that in cancer cells under nutrient starvation, transcriptional induction of GS1 by FOXO3 resulted in autophagy and survival with the concomitant inhibition of the TOR pathway [64]. We do not know at the moment if this pathway may be responsible for any GS1 activation in neurons, but we found that the expression of *Drosophila* FOXO in whole larvae transcriptionally induces *dGS1-mRNA*, suggesting that this regulation is conserved in flies (Supplementary Figure S5).

Expression of GS1 also reduces the level of glutamate, a nonessential amino acid that functions as an excitatory neurotransmitter in the central nervous system, and acts as a substrate in many distinct biochemical reactions [65]. Glutamate is synthesized from glutamine, α -ketoglutarate and 5-oxoproline as a precursor for the biosynthesis of various amino acids including proline (Pro) and arginine (Arg) [66,67]. Interestingly, in the heads of animals expressing GS1, we found reduced Pro and Arg, both relevant in the mechanism of TOR activation through specific amino acid sensors [58]. In particular, the lysosomal amino acid transporter SLC38A9 was proposed to be necessary for TOR activation by Arg in mammalian epithelial cells [68]. So far, there are no data describing the mechanism of activation by SLC38A9 in neurons. However, since we found Arg levels reduced in

the heads of animals expressing GS1, while Gln levels were high, we speculate that the Arg activation of SLC38A9 in neurons may act downstream of Gln in the stimulation of the TOR pathway, since Gln is not a limit.

It is worthy to note that, although the *Drosophila* SLC38A9 transporter has not been characterized yet, the *Drosophila* genome contains three genes expressed in the brain (CG13743, CG30394 and CG320181) [69–71], whose sequences are significantly homologous to that of human SLC38A9 [72], suggesting that they may encode for the fly functional homolog(s) of SLC38A9.

Our data also show that GS1 increases the level of Asn while Asp was decreased, suggesting an upregulation of asparagine synthetase (ASNS), a transaminase that is induced during nutrient starvation to indirectly synthesize amino acids to sustain protein synthesis [23,68,73,74]. Similarly, we saw as the levels of alanine (Ala) increased, suggesting the activation of alanine synthetase, another transaminase, whose level of activation may increase when glutaminolysis is high, in order to produce Glu from Gln to support metabolic pathways in the biosynthesis of proteins [75].

Finally, we analyzed the level of autophagy in human fibroblasts derived from healthy donors or patients carrying the mutation in the *huntingtin* gene (mHtt). These experiments revealed that fibroblasts carrying the mHtt suffer of a defect in the expression and possible cleavage of the LC3, thus suggesting that autophagy might be impaired in these cells. These data allow us to assume that autophagy may be defective also in post-mitotic, primary HD neurons; we would like to carry experiments to validate this hypothesis in the near future, using neuronal cells derived from iPSCs cells from donors and from patients carrying different CAGs mutations in the *huntingtin* gene.

In summary, malfunctions in the components of the glutamate–glutamine cycle have been detected in many neurodegenerative diseases, including Huntington disease [50,76,77]. Here we show a novel function for GS1, a member of the GGC, to control autophagy in post-mitotic cells, like neurons that are highly dependent on the endo–lysosomal pathway for their functional homeostasis. The function of GS1 in neurons may be important for the control of essential amino acids, including the switch to increase Asn, indicating a role in the biochemical homeostasis of neurons that maintain autophagy active. Here we can speculate that this function for GS1 in controlling autophagy is conserved also in humans, as fibroblasts from HD patients have defective autophagy due to the reduced level of LC3 accompanied by a reduction in GS1 levels.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1; Supplementary Figure S1: *ClustalW* amino-acid sequence alignment of *Drosophila* GS1 (UniProt E1JHQ1) and of human GLUL (UniProt P15104); Supplementary.; Figure S2: Reduction of GS1 enhances retinal defects induced by *Htt-Q93*; Supplementary.; Figure S3: Expression of *GS1* rescues neuronal death induced by *Htt-Q93*; Supplementary.; Figure S4: Schematic representation of the N-terminus region of the huntingtin protein and time line of the advent of the motility defects induced by *Htt-Q93*; Supplementary.; Figure S5: Expression of *GS1* induces *FOXO-mRNA*; Supplementary.; Figure S6: In adult males, co-expression of *GS1* with *Httex1-Q93* in neurons partially rescues animal survival (A) but not climbing defects (B) induced by *elav^{c155}-Httex1-Q93* in adult males.; Supplementary Figure S7: MSO (methionine sulfoximine) specifically inhibits GS1 activity in lysates from heads of *elav^{c155/+} and elav^{c155/+}; E[P] GS1^{G3347}* females.; Supplementary.; Figure S8: Co-expression of *GS1* with *Htt-Q93* in neurons partially rescues the climbing defects induced by expression of *elav^{c155}-Htt-Q93* visible in adult females, using two others independent recombinant *Htt-Q93*, *GS1* lines.; Supplementary Figure S9: Efficiency of *Atg1-RNAi* and *Atg5-IR 24-1* in reducing autophagic puncta and the relative protein expression in adult heads. Supplementary Figure S10: Expression of *elav-Atg8-mCherry* in the calyx of third instar larval brains. Supplementary Excel-S1: ANOVA analysis; Supplementary Video S1: larvae q16 ctl 25 sec; Supplementary Video S2: larvae q93 25 sec; Supplementary Video S3: adult climbing *Elav-Ex1-HttQ93* and Q16.

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